Department of the Navy
Office of the Chief of Naval Research
Grant No. N00014-90-J-1648
Principal Investigator: Thomas G. Burke, Ph.D.
Final Triannual Report, 1990

Evaluation of Liposome-Encapsulated Hemoglobin/LR16 Formulation as a Potential Blood Substitute

PERFORMANCE REPORT

Reducing the oxygen affinity of liposome-encapsulated hemoglobin (LEH) to a physiologically useful range is a key step in the development of an LEH product that may potentially be used as a blood replacement fluid. Our approach to this challenge is a pharmacological one, that being the development of drug molecules capable of lowering the oxygen affinity of purified hemoglobin. In our initial proposal, we presented data which demonstrated the ability of the phenylureido-substituted phenoxyisobutyric acid compound (LR16) to effectively modulate the oxygen affinity of purified human hemoglobin microencapsulated in lipid vesicles. For the surrogate blood application, LR16 appears to be of greater utility than hemoglobin's natural effector molecule 2,3-bisphosphoglycerate (DPG) because the latter compound undergoes fairly rapid hydrolysis during storage, thereby adversely affecting the integrity of the product.

In our previous performance report we presented experimental data concerning the ability of LR16 to modulate the oxygen binding properties of LEH. We showed that LR16 was a potent modulator of the oxygen-binding properties of LEH. LR16 at a concentration of 1.25 mM was found to shift the P_{50} value of LEH all the way out to a value of 30. LR16 was found to modulate the P_{50} value of LEH in a dose-dependant manner, thereby documenting that our pharmacological approach provides a means of altering systematically the P_{50} values of LEH formulations.

For example, a P_{50} value of 8.5 mm Hg was observed for purified hemoglobin (stripped of DPG) and encapsulated in the liposomes. Upon addition of LR16 to the hemosome suspension, the P_{50} value was right-shifted, and the changes observed in the P_{50} values occurred in a manner which was dependant upon the amount of LR16 added. The addition of 0.1 mM LR16 resulted in a shift in P_{50} to a value of 10, whereas the addition of 1.25 mM LR16 resulted in a shift much further out to a P_{50} value of 30.

As pointed out in our initial grant application, specific milestones for year 1 include the formulation and characterization of LEH/LR16 preparations which maintain a high concentration of hemoglobin (20 mM) and P_{50} values in the 30-40 mm Hg range. Other goals include sterile filtration and size characterization and optimization of the product.

To this end, our research efforts during this second four-month period of the initial year of funding of this grant has been focused in the following two areas:

1) continued physical characterization of the LEH/LR16 artificial blood formulation and 2) scale-up of the synthesis of LR16 and related analogues. Each of these aspects of our research program are described in greater detail below.

RESULTS

Physical Characterization of the LEH/LR16 Formulation

In the previous performance report concerning this grant we presented data concerning the ability of the drug LR16 to modulate the oxygen binding properties of LEH. In more recent experiments we have examined the effect of temperature on the ability of LR16 to modulate the oxygen binding properties of LEH. Shown in Table I are P_{50} values (in mm Hg) for LEH/LR16 formulations at various temperatures. Not only was temperature varied in this set of experiments, but the concentration of LR16 was varied as well. At each temperature, LR16 was capable of modulating the P_{50} values of LEH in a dose-dependant manner. Whereas the P_{50} value for LEH increased 2.4-fold over control upon the addition of LR16 (1.25 mM) at 25° C, an approximately equivalent change was observed upon the addition of the same amount of LR16 at physiological temperature. These experiments demonstrate that the ability of the LR16 agent to modulate the oxygen binding properties of LEH is <u>not</u> strongly dependant upon temperature.

Table I: P_{50} Values (in mm Hg) for LEH/LR16 Formulation at Various Temperatures

	Temperature			
LEH Sample	25°C	30.5°C	37°C	40°C
Control without drug	6.5	7.5	12.2	11.5
With 0.5mM LR16	9.0	14.0	23.3	24.5
With 1.0mM LR16	13.5	20.5	31.5	32.5
With 1.25mM LR16	15.5	23.5	36.5	37.0

In another recent aspect of our research we have been employing various microscopic methodologies in order to better characterize our LEH material. Characterization of an artificial blood formulation at the microscopic level is important in order to be able to monitor changes in the integrity of a given preparation which may occur with time temperature, storage, etc. Much time and effort during this past period of support has been devoted to developing our expertise in the area of microscopy.

Shown in Figures 1 and 2 are micrographic images of one of our LEH preparations taken using a transmission election microscopic technique and a freeze-fracture electron

Figure 1. An LEH/LR16 suspension examined by thin section electron microscopy. Image shows two vesicles, top one is clearly unilamellar.

Figure 2. Electron micrograph of a freeze-fracture replica prepared from LEH/LR16 particle.

micrographic technique, respectively. The images present in Figure 1 are from a thin-sectioned sample of LEH, and this figure quite clearly shows the multilamellar nature of an LEH liposome. Figure 2 shows an election micrograph of a freeze-fracture replica of an LEH liposome, showing a close-up view of the outer bilayer of the vesicle. These micrographic images were prepared as described in the Experimental Section.

In the future, we plan to use these microscopic methodologies to follow changes induced in LEH formulations which occur as a result of sterile filtration, LR16 presence (to determine if LR16 promotes vesicle aggregation or fusion), temperature and storage. We will also evaluate how changes in lipid composition, an alteration known to affect circulation half-lives in vivo, modulates LEH integrity.

Our findings to date concerning LEH/LR16 are summarized in an abstract entitled: "Liposome-Encapsulated Hemoglobin: Use of LR16 Analogues in the Optimization of Its Oxygen Binding Properties" submitted to the 35th Annual Meeting of the Biophysical Society to be held from February 24-28, 1991 in San Francisco, CA. A copy of this abstract can be found at the back of this progress report.

Scale-Up of the Synthesis of LR16

In years 2 and 3 of this proposal we intend to produce LEH/LR16 in sufficiently large quantities in order to be able to evaluate the efficacy of the artificial blood material in small animals. In order to increase the amounts of LR16 available for use, we have recently scaled up the synthesis. This work was carried out by Dr. Alok Singh of the Naval Research Lab. From his experiments, Dr. Singh was able to produce a large 600 mg sample of an LR16 product.

Using HPLC methodologies, we have found that the recently-synthesized LR16 product contains an impurity. Chromatograms of an authentic sample of LR16 and that of the recently synthesized material both showed a single, rapidly-eluting peak with a retention time of 1.2 min. Spectral Analysis of these peaks (Isograms are shown in Figure 3 on the next page) demonstrate that the newly-synthesized LR16 product coelutes with an impurity (or impurities) which absorb strongly in the 260-280 nm region of the spectra. We are presently in the process of further purifying the LR16 material using an ion exchange chromatographic procedure.

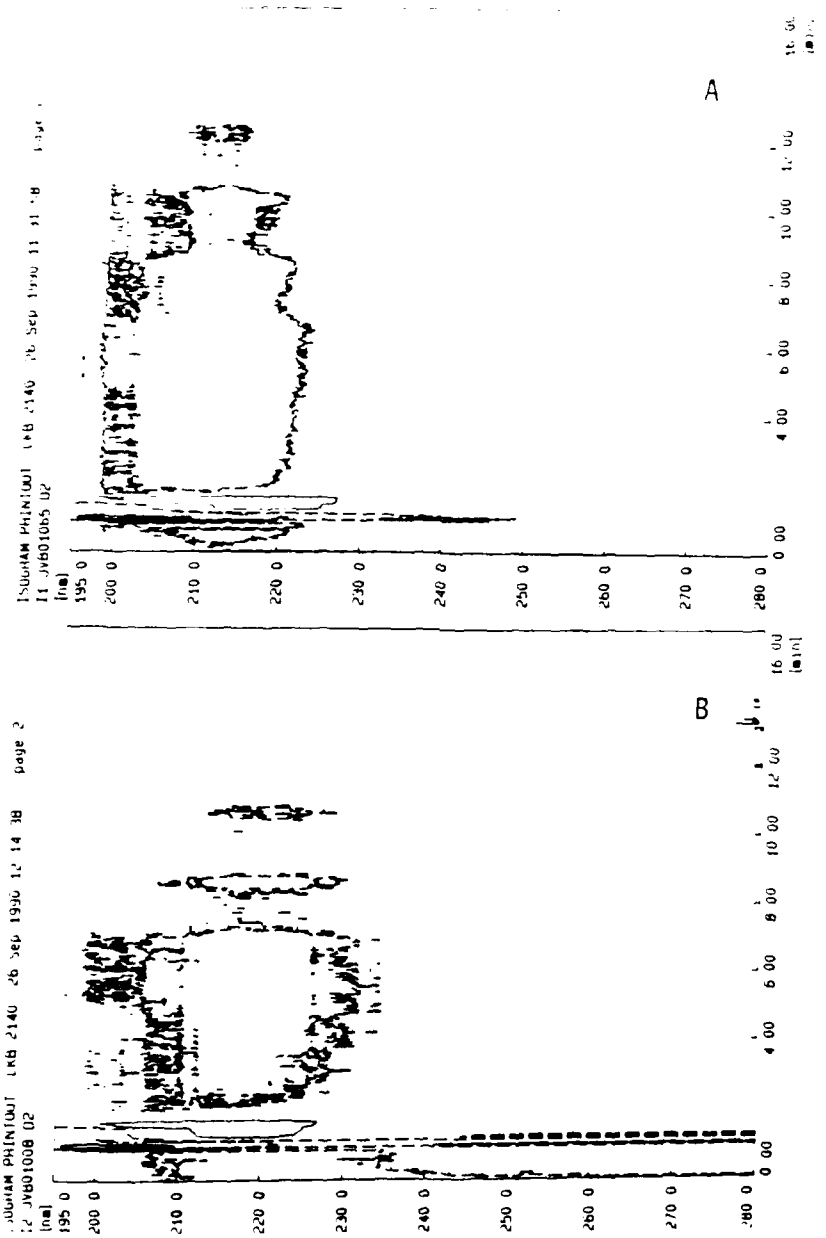

Figure 3. Isograms of an authentic sample of LR16 (panel A) compared to the sample recently obtained from a scaled-up synthesis (panel B). Note that the compound represented in isogram B contains and an impurity which absorbs between 250 nm and

EXPERIMENTAL METHODS

Preparation of Hemolysate

Hemoglobin solutions are prepared from outdated human blood washed with saline (0.9% NaCl in water), centrifuged for 10 minutes at 3,000 rpm, and lysed with cold distilled water. The sample is then centrifuged at high speed (16,000 rpm for 30 minutes) and dialyzed with 30 mM aqueous sodium phosphate (pH 7.4) at 4° C. This buffer is changed preferably four times during the procedure. An aqueous solution containing 5.6% by weight of hemoglobin is produced.

The hemoglobin is concentrated by pressure ultrafiltration through a 30,000 dalton cut-off filter. In this manner, a hemoglobin concentration of 17.7% by weight (2.75 mM) in water may be reached. Aqueous solutions containing at least about 10% by weight, preferably from about 15% to about 20% by weight, are sought.

Prior to encapsulation, the aqueous hemoglobin solution is again centrifuged at 16,000 rpm for 20 minutes to remove any newly precipitated material. Hemoglobin solutions are maintained at 5° C whenever possible throughout the course of this procedure.

Preparation of Liposome Encapsulated Hemoglobin/LEH

The synthetic membrane materials useful to encapsulate hemoglobin include a mixture of the following lipids: dimyristoyl phosphatidylcholine (DMPC), dipalmitoyl phosphatidylcholine (DPPC), distearoyl phosphatidylcholine (DSPC), cholesterol and dimyristoyl phosphatidylglycerol (DMPG). The ratios of lipids is preferably 1:1:1:2.7:0.3 for DMPC:DPPC:DSPC:cholesterol:DMPG, respectively. Other lipid mixtures, including those which contain longer chain phospholipids (See Gregoriadis (1984) in Liposome Technology, CRC Press, Boca Raton,Florida) may be used, e.g., to promote longer circulation half-life in vivo). Typically, a 17.5 mg amount of total lipid is weighed out and dissolved in 5 ml of chloroform. The lipid is then dried to a thin film in a round-bottom flask on a rotary evaporator and the flask is held under vacuum until all traces of residual chloroform are removed.

To the lipid film 1 ml of the concentrated hemoglobin solution (for example, 17% or 2.76 mM) is added, the sample is then vortexed and shaken at 37° C in a water bath for 1 hour. The hemoglobin-lipid suspension is placed at 20° C in an ultra-filtration cell (Amicon) and large unilamellar vesicles of relatively uniform size (mean diameter between 2-3 μm) are formed by successive extrusion through polycarbonate membrane filters of decreasing pore size (10, 5, and 2 um). The resulting suspension is centrifuged for 30 minutes at 16,000 rpm. The pellet is resuspended, washed twiced with phosphate buffered saline (PBS) (pH 7.4) for 20 minutes at 16,000 rpm. The size of the LEH vesicles ranges from about 1.5 μm to about 3 μm.

Incorporation of LR16 into LEH

Incorporation of the LR16 into the LEH may be accomplished in two ways. The LR16 may be simultaneously coencapsulated with the hemoglobin by inclusion in the concentrated hemoglobin solution prior to vortexing or ultrafiltration. An aqueous concentration of LR16 from about 0.004% by weight to about 0.05% by weight in the concentrated hemoglobin solution is appropriate.

Alternatively, the LEH may be suspended in a buffered aqueous LR16 solution which penetrates the membrane thus providing the desired coencapulation of hemoglobin and LR16.

A preferred solution may comprise from about 1 mM to about 0.1 mM LR16 in buffered aqueous solution having a pH of from about 7.35 to about 7.45. Suitable buffers include HEPES (N-2-hydroxyethylpeperazine-N'-2-ethane sulfonic acid sodium salt) or BIS-TRIS (bis [2-hydroxyethyl]imino-tris [hydroxymethyl]methane). One suitable buffer solution contains about 100 mM HEPES and about 0.9% of sodium chloride.

The LEH is suspended in the buffered LR16 solution for a time period appropriate to achieve the desired modulation of the oxygen affinity of the encapsulated hemoglobin. Suspension from about 36.5° C to 37.5° C for a time period of about 30 minutes is appropriate.

Determination of LEH P_{50} Values

P_{50} values were determined in a Hemox Analyzer (TCS, Southhampton, PA). This analyzer was equipped with a thermostated cuvet compartment.

Freeze Fracture Analyses.

Samples of liposomes were mixed with an equal volume of 20% glycerol, transferred to a sample holder and frozen in liquid dichlorodifluoromethane -(CCl_2F_2 Dupont Freon 12) and then immersed in liquified nitrogen. The frozen samples were transferred to the Ladd Vacuum Evaporator which was equipped with a precooled freeze fracture unit, fractured, shadowed with platinum-carbon, and coated with carbon. Replicas were floated off into distilled water and subjected to 20% sodium hypochlorite, washed in distilled water, picked up on formvar coated grids and examined in the Philips CM 10 electron microscope.

Transmission Electron Microscopy

Samples of liposomes were fixed in 2% cacodylate buffered glutaraldehyde (pH=7.4) for 3 hours, gently spun in a centrifuge to form a pellet, washed in cacodylate buffer, post-fixed in 2% osmium tetroxide for 1 hour, washed in buffer, dehydrated through graded series of alcohols into propylene oxide, embedded in Epon/Araldite, sectioned at 60nm on an LKB Ultrotome III, stained with uranyl acetate and lead citrate and examined in the Philips CM 10 electron microscope.

HPLC Chromatographic Analysis of Drug Purity

Chromatographic analysis of LR16 was achieved on using a modular chromatographic system consisting of an LKB Bromma Model 2249 gradient pump, an LKB Bromma Model 2140 ultraviolet diode array detector, and a Rheodyne injector with a 20 μl injection loop. The HPLC was interfaced to an IBM-compatible computer with a Model 80286 microprocessor and a 60 megabyte fixed disk drive. Data analysis was accomplished using LKB Bromma Wavescan enhanced graphics software. A reversed-phase LKB Spherisorb OBS2 (3 μm particle size) Superpac Column was used with a mobile phase consisting of pH 4.0 ammonium formate buffer (flow rate of 1 ml/min.).

Corresponding Author Thomas G. Burke, Ph.D.

Telephone (818) 359-8111 ext. 4501

Member or Sponsor's Signature Thomas G. Burke

Typed or printed name Thomas G. Burke

☐ I am willing to chair a session. Tele _____

PRESENTATION CHOICE ☐ Oral ☒ Poster ☐ No preference
☐ Poster will require VCR. Author will be billed.

Category Codes
See reverse side for categories.

1st Choice 8J

2nd Choice _____

TOP

4. Deadline for Receipt of Abstracts: October 15, 1990. See mailing instructions under item 6. Mailing address: Biophysical Society Office, 9650 Rockville Pike, Bethesda, MD 20814.

5. Abstract Preparation: The block will be reduced by 36% for printing in the abstract volume. Only typing within the blue lines will be printed. Do not leave top or bottom margins within the rectangle. Single space all typing. Use a typewriter with clean ELITE type. Use carbon ribbon or a new black silk ribbon. Abstracts typed with a low-quality dot-matrix printer do not print well. Hand print in BLACK ink any symbols not on your typewriter, using a fine-tipped pen, not felt-tipped.
Type title in full caps. If no author is a member type the phrase "(Intro. by...)" after the last author including the name of the sponsoring member. The sponsor must sign the abstract in lieu of appearing as primary author on another paper.
CORRECTIONS: Abstracts will not be copyedited. Proofread before mailing. Use self-adhesive correction tape for simple errors. Do not erase. If the abstract is marred in any substantial way, retype on a new form, not a photocopy of the form.

6. **Abstract Mailing: Send this form with four (4) photocopies. Enclose stamped, self-addressed acknowledgement and assignment cards. Please do not use staples on any cards. Do not fold forms.** IMPORTANT: Copies of all correspondence containing special instructions (e.g., a request to schedule two papers consecutively) must be stapled to all *photocopies* of the abstract form. Do not staple anything to the original form.

7. NO SUBSTITUTIONS OF ABSTRACTS CAN BE GUARANTEED. PLEASE PROOFREAD YOUR ABSTRACT. Abstracts may be withdrawn prior to October 26, 1990.

LIPOSOME-ENCAPSULATED HEMOGLOBIN: USE OF LR16 ANALOGUES IN THE OPTIMIZATION OF ITS OXYGEN BINDING PROPERTIES.

Thomas G. Burke, Yayesh Asmerom[+], Alok Singh[*], and Sam Rahbar[+], Dept. of Medical Oncology and Therapeutics Research, and [+]Department of Hematology and Bone Marrow Transplantation, City of Hope National Medical Center, Duarte, CA 91010 and [*]Center for Bio/Molecular Science and Engineering, Naval Research Laboratory, Washington, DC 20375

Reducing the oxygen affinity of liposome-encapsulated hemoglobin (LEH) to a physiologically-optimal range is a key step in the development of an LEH product that may potentially be used as a blood replacement fluid. We have shown that the phenylureido-substituted phenoxyisobutyric acid compound referred to as LR16 [Lalezari et al.(1988) PNAS 85, 617] is effective at modulating the oxygen affinity of purified human hemoglobin microencapsulated in lipid vesicles. In these experiments, hemoglobin was purified from outdated human blood and concentrated to 2.75 mM using pressure ultrafiltration. Hemoglobin solution (1 ml) and LR16 together were encapsulated in synthetic membrane materials (18 mg) consisting of DMPC, DPPC, DSPC, cholesterol and DMPG (ratios of 1:1:1:2.7:0.3, respectively). Liposomes of relatively uniform size (mean diameter between 2-3 um as determined by freeze fracture electron microscopy) were formed by successive extrusions through polycarbonate membrane filters of decreasing pore size. Whereas the P_{50} value for LEH in the absence of drug was 9 mm Hg, the inclusion of LR16 at concentrations of 0.1 mM, 0.2 mM, 0.5 mM, 1 mM, and 1.25 mM resulted in higher P_{50} values of 10 mm Hg, 13 mm Hg, 19 mm Hg, 27 mm Hg and 30 mm Hg, respectively. Thus, the presence of LR16 allows the oxygen dissociation curve of LEH to be right-shifted to a physiologically more relevant P_{50} range. Such a pharmacological approach provides a means of alternating systematically the P_{50} values of LEH formulations. This work was supported by Office of Naval Research Grant No. N00014-90-J-1648 to TGB.

8. Overhead and 2"x2" projectors will be provided in all oral scientific sessions. For VCR equipment, check the appropriate box on the abstract form. Posters using VCR's will be placed in one section of poster boards. Every effort will be made to meet special equipment requests, however, the author will be billed. To request special equipment or to determine approximate cost, contact Ms. Marcia Sternburg (301) 530-7010, prior to December 2[illegible] 1990.